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Calystegins, a Novel Class of Alkaloid Glycosidase Inhibitors

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The alkaloid extract from roots of naturally growing *Convolvulus arvensis*, purified by ion-exchange chromatography, showed significant inhibitory activity toward β-glucosidase and α-galactosidase. The demonstrated occurrence of polyhydroxy-*nor*tropane alkaloids, the calystegins, in *C. arvensis* and their structural similarity to known polyhydroxy alkaloid glycosidase inhibitors, suggested that these might be responsible for the observed activity. Pure calystegins, isolated from transformed root cultures of the related plant species *Calystegia sepium*, were tested for glycosidase inhibitory activity. The purity of the alkaloids was established by gas chromatography and their identity confirmed by their mass spectrometric fragmentation patterns. The trihydroxy alkaloid, calystegin A_3, was a moderately good inhibitor of β-glucosidase ($K_i = 4.3 \times 10^{-5}$ M) and a weak inhibitor of α-galactosidase ($K_i = 1.9 \times 10^{-4}$ M). An increased level of hydroxylation, as in the tetrahydroxy calystegins B, consisting of 27% calystegin B_1 and 73% calystegin B_2, resulted in greatly enhanced inhibitory activity. The calystegins B were potent inhibitors of β-glucosidase ($K_i = 3 \times 10^{-6}$ M) and α-galactosidase ($K_i = 7 \times 10^{-6}$ M). These levels of activity are comparable with those of the polyhydroxy indolizidine alkaloids castanospermine and swainsonine toward α-glucosidase and α-mannosidase, respectively, and of the polyhydroxy pyrrolizidine alkaloid australine toward α-glucosidase. The calystegins therefore compose a new structural class of polyhydroxy alkaloids, the *nor*tropanes, possessing potent glycosidase inhibitory properties. © 1993 Academic Press, Inc.

The isolation of a novel group of alkaloids named calystegins from roots and root exudates of *Calystegia sepium*, a member of the plant family Convolvulaceae, has recently been reported (1). The same, or closely related compounds, have also been shown to be present in roots of the related species *Convolvulus arvensis*, as well as in *Atropa belladonna*, a member of the Solanaceae family. Three of the six calystegins shown to be present in *Ca. sepium* have been structurally characterized as polyhydroxy-*nor*tropane alkaloids by the use of ^1H and ^{13}C NMR spectroscopy and mass spectrometry (2, 3). Calystegin A_3 was identified as the trihydroxy tropane (**1**) (Fig. 1), whereas calystegins B_1 and B_2 were established as the isomeric tetrahydroxy tropanes (**2**) and (**3**), respectively. The calystegins therefore constitute a unique subgroup of the tropane alkaloid class, characterized by the absence of an N-methyl substituent and the high degree of hydroxylation. The closest structural relative to the calystegins is the 2-hydroxy-6-acetoxy-*nor*tropane alkaloid, Bao Gong Teng A, isolated from the Chinese herb *Erycibe obtusifolia*, which has shown some promise for the treatment of glaucoma (4).

Polyhydroxy alkaloids, encompassing several structurally related classes, have been isolated from both plants and microorganisms in steadily increasing numbers over the past decade. These natural products have been found to be competitive inhibitors of various glycosidases, presumably by virtue of structural similarities to carbohydrates (5, 6). The more potent members of this group have been employed as biochemical tools with which to investigate the effect of altered glycosylation patterns, caused by inhibition of processing enzymes, on the structure and function of glycoproteins (7, 8). They have also been shown to exhibit antiviral (9–12), antimetastatic (13), antihyperglycemic (14), and immunostimulatory activity (15), as well as the ability to alter glycogen metabolism in animals (16).

Four structural classes of polyhydroxy alkaloid glycosidase inhibitors are currently known. These are the monocyclic pyrrolidines and piperidines represented by dihydroxymethyldihydroxypyrrolidine (**4**) (17) and deoxynojirimycin (**5**) (18), respectively (Fig. 1), and the bi-

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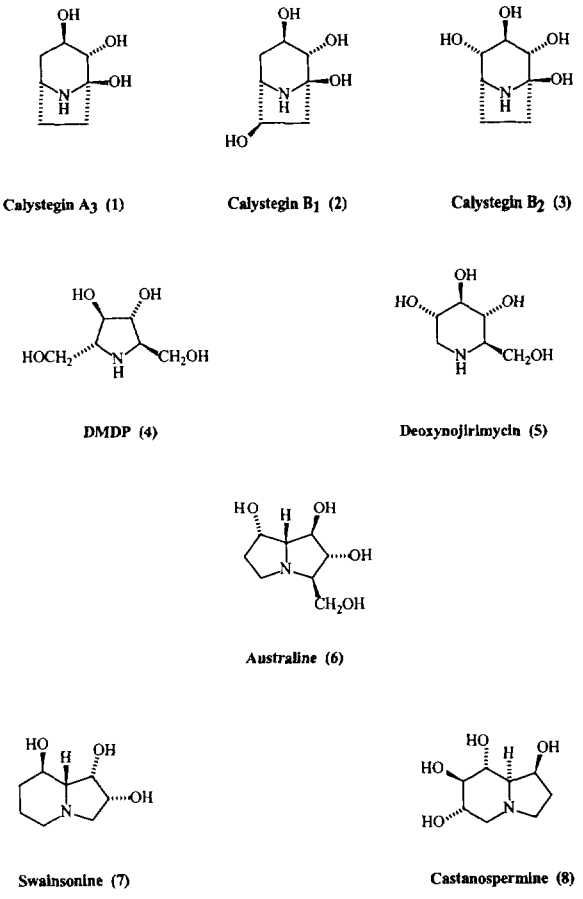

FIG. 1. Structures of the polyhydroxy-*nor*tropane alkaloids, calystegins A_3, B_1, and B_2, and of structurally related polyhydroxy pyrrolidine, piperidine, indolizidine, and pyrrolizidine alkaloid glycosidase inhibitors. The calystegins are represented in a planar form to emphasize the structural affinities to the mono- and bi-cyclic alkaloids. The full stereochemical representation of the calystegins is illustrated in Fig. 4.

cyclic pyrrolizidines and indolizidines. Polyhydroxy pyrrolizidines such as australine (**6**) (19) may be regarded from a structural point of view as two fused pyrrolidine ring systems, whereas polyhydroxy indolizidines such as swainsonine (**7**) (20, 21) and castanospermine (**8**) (22) can be considered to be a fused hybrid of pyrrolidine and piperidine rings.

The calystegins represent a novel class of bicyclic polyhydroxy alkaloids with structural affinities to polyhydroxy indolizidines and piperidines. These structural analogies to known glycosidase inhibitors suggested that the calystegins might have similar properties. In this paper we report the potent β-glucosidase and α-galactosidase inhibitory activity of calystegins A and B, establishing them as a fifth structural class of polyhydroxy glycosidase inhibitors. Calystegins B exhibited significantly higher activity than calystegin A, indicating a correlation of enhanced potency with increasing degree of hydroxylation, comparable with that observed for castanospermine and its relatives. We also report a gas chromatographic–mass spectrometric (GC-MS) method for the analysis and structural identification of the calystegins as their trimethylsilyl (TMS)[2] derivatives.

EXPERIMENTAL PROCEDURES

Materials. The enzymes amyloglucosidase (from *Aspergillus niger*), β-glucosidase (from almonds), α-galactosidase (from *A. niger*), β-galactosidase (from bovine liver), α-L-fucosidase (from bovine kidney), β-xylosidase (from *A. niger*), and α-mannosidase (from jack beans) and all the *p*-nitrophenyl glycoside substrates were obtained from Sigma Chemical Co. β-Mannosidase was purified from *A. niger* as previously described (23).

Enzyme assays. The enzymatic activities of amyloglucosidase, β-glucosidase, α- and β-galactosidase, α- and β-mannosidase, α-L-fucosidase, and β-xylosidase were determined colorimetrically by monitoring the release of *p*-nitrophenol from the appropriate *p*-nitrophenyl glycoside substrate (24). All reaction mixtures contained 50 μmol of sodium acetate buffer, pH 5.0, 1.5 μmol of *p*-nitrophenyl glycoside, and enzyme, in a final volume of 0.5 ml. Incubations were for 15–60 min at 37°C, and the reactions were terminated by the addition of 2.5 ml of 0.4 M glycine buffer, pH 10.4. The *p*-nitrophenol liberated in the reaction was measured at 410 nm. Assays were done under conditions where the amount of *p*-nitrophenol released was linear with both time and protein concentration.

Extraction of calystegins from C. arvensis. Mature *C. arvensis* was collected from an uncultivated area on the grounds of the Western Regional Research Center (Albany, CA). The roots were separated from the aboveground parts of the plant, chopped coarsely, air-dried, and then ground to pass a 1-mm screen. This material (95 g) was extracted with methanol in a Soxhlet apparatus for 3 days and the extract was purified by ion-exchange chromatography on a Dowex 50W X8 resin, as previously described for the polyhydroxylated alkaloids of *Castanospermum australe* (25). On concentration, the ammonium hydroxide eluate deposited a white crystalline solid (0.1 g, 0.12% yield). The residue obtained on evaporation to dryness was freeze-dried to give the alkaloid fraction as a brown, friable solid (1.83 g, 1.93% yield).

Isolation of Calystegins A and B. Calystegins were isolated from an aqueous extract of a transformed root culture of *Ca. sepium* as previously described (1). The alkaloids were then purified by ion-exchange chromatography, and basic amino acids were removed by treatment with *Agrobacterium tumefaciens*. Calystegins A and B were separated by filtration on a GF05 column (IBF) (2). The calystegin A and calystegin B fractions were analyzed for purity by GC-MS of their TMS derivatives.

Gas chromatography–mass spectrometry of calystegins. Alkaloid fractions were analyzed for homogeneity by GC-MS of the TMS derivatives, prepared by treatment with *N*-methyl-*N*-(trimethylsilyl)-fluoracetamide (MSTFA) in pyridine at 60°C for 12 h. A Hewlett–Packard 5890 Series II gas chromatograph, equipped with a 5971 mass selective detector operating at 70 eV, on-column injector, and 60-m × 0.32-mm i.d. SE-30 fused silica column was used for the analysis. The column was temperature programmed from 120 to 300°C at 10°/min.

RESULTS

Isolation of Alkaloid Fraction from C. arvensis

Purification of the crude methanolic extract of the roots of naturally growing *C. arvensis* by chromatography on a Dowex 50 NH_4^+ ion-exchange column gave a fraction consisting of alkaloids and other basic components, on elution with dilute ammonium hydroxide. Concentration of the eluate gave a white, crystalline solid in 0.12% yield of the

[2] Abbreviations used: TMS, trimethylsilyl; MSTFA, *N*-methyl-*N*-(trimethylsilyl)-fluoracetamide.

dry weight of plant material, which was shown by GC-MS of its TMS derivative to be asparagine (data not shown). Evaporation of the eluate to dryness, followed by freeze-drying, gave the crude alkaloid fraction as a brown, hygroscopic solid in 1.93% yield. This material was screened for glycosidase inhibitory activity (vide infra).

Isolation and Analysis of Calystegins A and B from Ca. sepium

The aqueous extract of transformed root cultures of *Ca. sepium* was purified and separated by ion-exchange chromatography and gel filtration as previously described (2).

Analysis of the calystegin A fraction by GC-MS showed the presence of a single component with a retention time of 15.55 min (Fig. 2a). The mass spectrum of this compound was consistent with the structure of the tetra-TMS derivative of calystegin A_3 (**1**). The calystegin B fraction exhibited two peaks eluting at 16.51 (27%) and 18.11 (73%) min (Fig. 2b). The mass spectra of these components were consistent with penta-TMS derivatives of calystegin B_1 (**2**) and calystegin B_2 (**3**), respectively. The calystegin A and calystegin B fractions were assayed for glycosidase inhibitory activity (vide infra).

Mass Spectral Fragmentation and Identification of Calystegin TMS Derivatives

The single component of the calystegin A fraction from *Ca. sepium,* with a GC retention time of 15.55 min, gave a molecular ion at m/z 447 (Fig. 3a). This ion corresponds to a tetra-TMS derivative of calystegin A_3 (**1**). A peak of similar intensity occurred at m/z 432, showing a loss of 15 amu from the molecular ion, a characteristic fragmentation of TMS derivatives caused by loss of a $-CH_3$ moiety. The base peak in the mass spectrum occurred at m/z 229 with another major fragment ion at m/z 358 (Fig. 3a). Analysis of the mass spectrum in relation to the established fragmentation pattern of underivatized tropane alkaloids (26, 27) indicates that calystegin A_3 tetra-TMS derivative (**1a,** Fig. 4) undergoes an analogous fragmentation. Thus the base peak at m/z 229 is due to the dihydropyrrolinium ion (**1d**) generated by cleavage of the six-membered ring portion of the tropane structure via the tricyclic ion (**1b**) and the 2-substituted dihydropyrrolinium ion (**1c,** m/z 243).

The major component of the calystegin B fraction isolated from *Ca. sepium,* with a GC retention time of 18.12 min, gave a molecular ion at m/z 535 (Fig. 3c) corresponding to a penta-TMS derivative (**3a**) (Fig. 4). The mass spectrum showed a base peak at m/z 229, identical to that in the mass spectrum of calystegin A_3, indicating that the compound was calystegin B_2, since the additional hydroxyl group had to be located in the six-membered ring which undergoes fragmentation. However, the 2-substituted dihydropyrrolinium ion (**3c**) resulted in a fragment with m/z 331 due to the presence of this extra hydroxyl group.

The minor component of the calystegin B fraction was also a penta-TMS derivative with a molecular ion at m/z 535, but exhibited a base peak at m/z 317 with additional major fragment ions at m/z 446 and 331 (Fig. 3b). The base peak must therefore be due to a dihydropyrrolinium ion (**2d**) bearing one more hydroxyl group than that arising from calystegins A_3 and B_2. Such a fragment can only be accomodated if the minor component is calystegin B_1. The peaks at m/z 446 and 331 are due to the tricyclic ion (**2b**) and the 2-substituted dihydropyrrolinium ion (**2c**), respectively (Fig. 4).

Glycosidase-Inhibitory Activity of Calystegins

Preliminary screening of the crude alkaloid extract of *C. arvensis* against a series of glycosidases showed that the extract had significant inhibitory activity toward β-glucosidase and α-galactosidase, but not toward any of the other enzymes tested. Since structural similarities to polyhydroxy alkaloid glycosidase inhibitors such as castanospermine and australine suggested that the inhibitory activity of the extract resided in the calystegins, calystegin A and B fractions isolated from *Ca. sepium* were assayed for inhibitory activity.

Calystegin A_3 was found to be a good inhibitor of β-glucosidase, showing 50% inhibition at 6.7 μg/ml. The alkaloid was also a fairly good inhibitor of α-galactosidase with 50% inhibition occurring at 30 μg/ml. The calystegin B complex, composed of 27% B_1 and 73% B_2, was much more potent, showing 50% inhibition of β-glucosidase at 0.6 μg/ml and of α-galactosidase at 1.2 μg/ml. Figure 5 shows a concentration curve comparing the degree of inhibition of β-glucosidase and α-galactosidase by various amounts of calystegin A_3 and the calystegins B. Neither of the calystegin fractions showed any inhibitory activity toward α-glucosidase, α- or β-mannosidase, β-galactosidase, α-fucosidase, or β-xylosidase when tested at a concentration of 20 μg/ml.

In order to establish whether the calystegins were competitive or noncompetitive inhibitors of β-glucosidase and α-galactosidase, a series of experiments were done in which the *p*-nitrophenyl glycosidase substrate concentration was varied, and several different concentrations of the respective alkaloids were used. When the data from these experiments was plotted by the method of Lineweaver and Burk, the intercept of $1/V$ versus $1/S$ was the same in the presence or absence of the inhibitor, indicating that the inhibition is of the competitive type (Fig. 6).

DISCUSSION

The calystegins, a novel class of polyhydroxy-*nor*tropane alkaloids, were first isolated and structurally characterized from transformed root extracts of *Ca. sepium* but were also shown to be present in the roots of *C. arvensis* and *A. belladonna* by analysis of crude extracts using high voltage electrophoresis and silver staining (1). The abundance of naturally growing *C. arvensis* (field

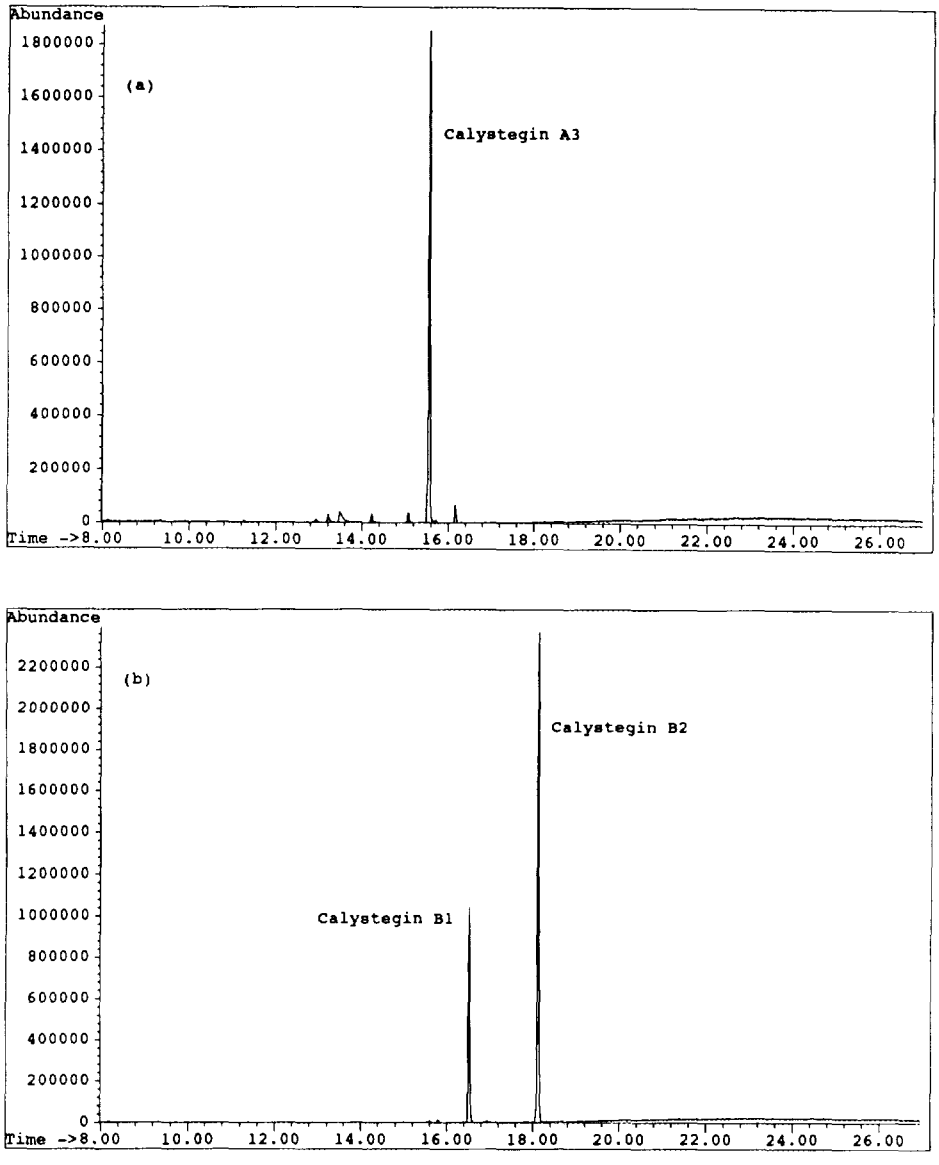

FIG. 2. Gas chromatographic analysis of TMS derivatives of: (a) calystegin A and (b) calystegin B. Separations were performed on a 60-m × 0.32-mm i.d. SE-30 fused silica column, temperature programmed from 120 to 300°C at 10°C/min.

bindweed), a common weed, provided an opportunity to prepare crude extracts to screen for glycosidase inhibition in order to test our hypothesis that the structural similarities of the calystegins to other classes of bicyclic alkaloids such as the polyhydroxy indolizidine and pyrrolizidine alkaloids might give rise to a similar type of biological activity. These initial screening assays indicated that the calystegin-containing extract showed significant inhibitory activity toward β-glucosidase and α-galactosidase.

It therefore became of interest to test the inhibitory activity of pure calystegins, and inhibition assays were performed on small samples of the alkaloids remaining from their original isolation from *Ca. sepium* root cultures. The availability of calystegins A and B provided an opportunity to determine not only the type and specificity of the inhibition but also the relationship of the greater degree of hydroxylation in the calystegins B relative to calystegins A to the inhibitory potency of the individual alkaloids. The results presented herein describe not only these biological activities but also the development of a gas chromatographic–mass spectrometric method for the analysis and structural identification of calystegins.

Treatment of the calystegins with MSTFA for a period of 12 h resulted in pertrimethylsilylation, whereas short reaction times resulted in partial derivatization. The calystegin A fraction isolated from *Ca. sepium* gave a single peak on GC analysis and inspection of the mass spectrum showed it to be a tetra-TMS derivative with a fragmentation pattern consistent with calystegin A_3 (**1**). The ca-

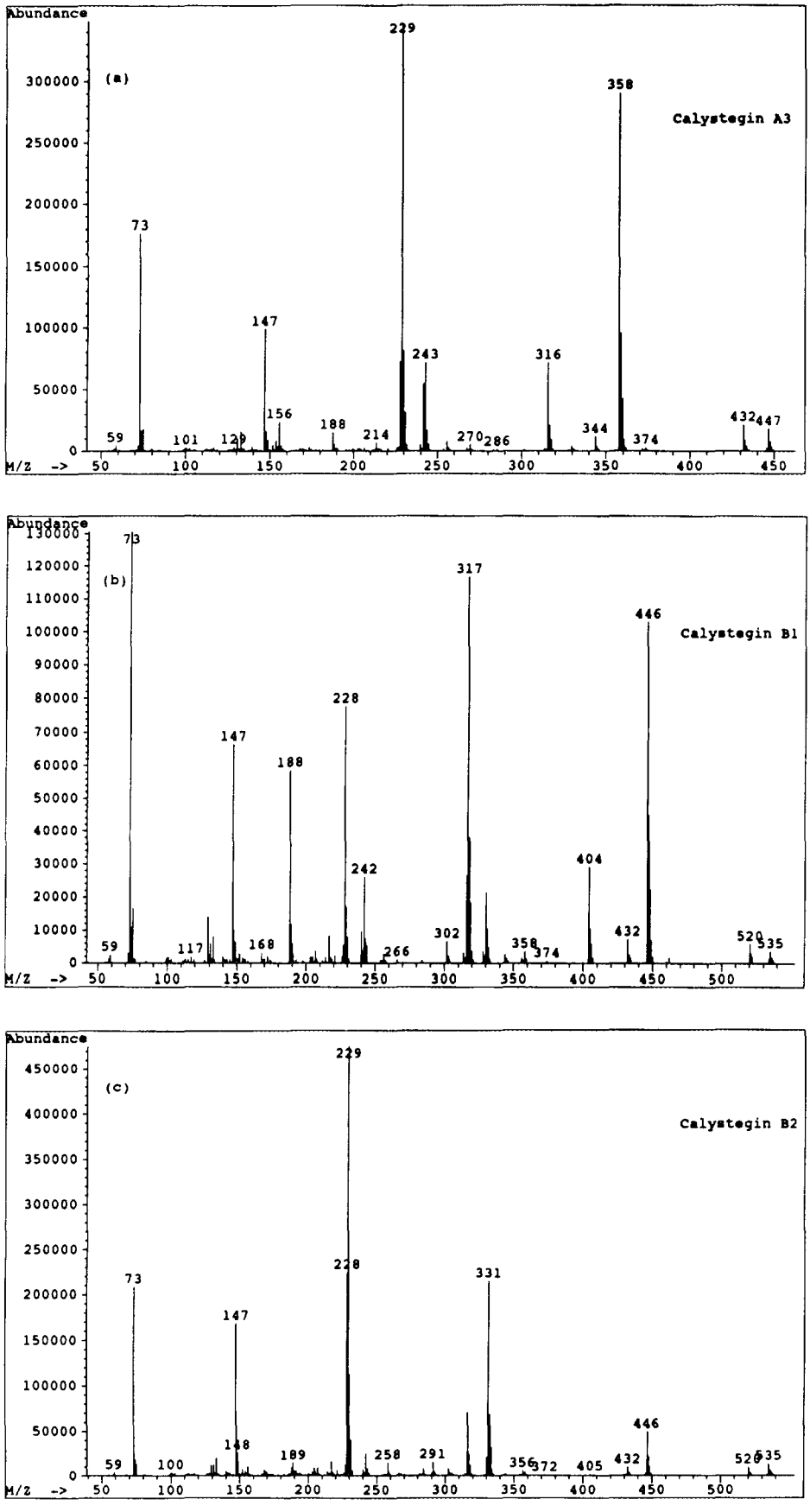

FIG. 3. Mass spectra of TMS derivatives of: (a) calystegin A_3; (b) calystegin B_1; (c) calystegin B_2. Spectra were determined at 70 eV in the EI mode.

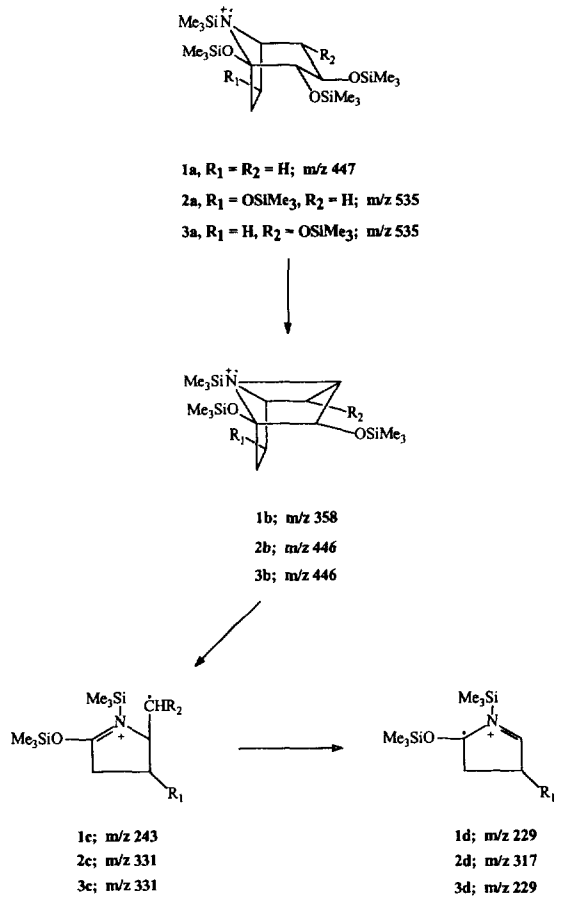

FIG. 4. Mass spectrometric fragmentation of TMS derivatives of calystegins: (**1a–1d**) calystegin A_3; (**2a–2d**) calystegin B_1; (**3a–3d**) calystegin B_2.

lystegin B fraction was shown to be a mixture of two penta-TMS derivatives consisting of 73% of a major component and 27% of a minor component. The mass spectral fragmentation pattern of each of these derivatives was unequivocal in establishing the position of the extra hydroxyl group relative to calystegin A_3 and showed that the major constituent was calystegin B_2 (**3**) and the minor constituent was calystegin B_1 (**2**).

The majority of naturally occurring polyhydroxy bicyclic alkaloids have been found to be inhibitors of amyloglucosidase, an α-glucosidase (28). The most potent of these are castanospermine, which produces 50% inhibition of the enzyme at 8 μM (29, 30), and australine, which produces the same effect at 6 μM (31). Epimerization of the hydroxyl substituents results in a decrease in the inhibitory activity. For example, 6-*epi*castanospermine shows 50% inhibition of amyloglucosidase at 20 μM (25). In addition to inhibition of amyloglucosidase, castanospermine also inhibits β-glucosidase, a concentration of 53 μM being necessary to produce 50% inhibition. Swainsonine is unique among this group of alkaloids in being a potent inhibitor of α-mannosidase, with a 50% inhibition concentration of 2 μM (32).

In contrast to castanospermine and australine, neither calystegin A_3 nor the calystegins B are inhibitors of amyloglucosidase. However, both inhibit β-glucosidase, calystegin A_3 being a moderately good inhibitor with a K_i of 43 μM, while the calystegin B complex is a very potent inhibitor of the enzyme, having a K_i of 3 μM. In addition both alkaloids inhibit α-galactosidase. Whereas calystegin A_3 is a relatively weak inhibitor of the latter, with a K_i of 190 μM, the calystegin B fraction is a potent inhibitor, with a K_i of 7 μM. The inhibitory activity of the calystegins B toward β-glucosidase and α-galactosidase is therefore comparable to that of castanospermine and australine toward amyloglucosidase, and of swainsonine toward α-mannosidase. In the absence of individual samples of calystegin B_1 and calystegin B_2 with which to perform inhibition assays, it is not possible to determine whether the potency of inhibition differs significantly between the two alkaloids. It appears probable, however, that each of

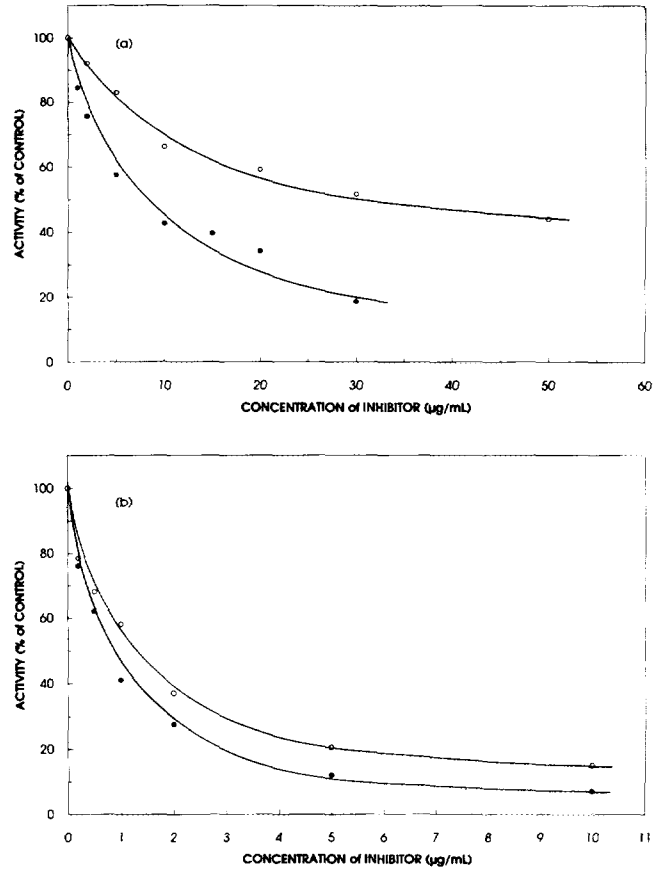

FIG. 5. Effects of (a) calystegin A and (b) calystegin B concentrations on the activity of β-glucosidase and α-galactosidase. Incubation mixtures contained 50 μmol acetate buffer, pH 5.0, 1.5 μmol *p*-nitrophenyl glycoside and various amounts of inhibitors, calystegin A or calystegin B, in a final volume of 0.5 ml. Reactions were terminated by the addition of 2.5 ml of 0.4 M glycine buffer, pH 10.4, and the amount of *p*-nitrophenol released was measured at 410 nM. Results are presented as percentage of control. Curves are as follows: (solid symbols) β-glucosidase and (open symbols) α-galactosidase.

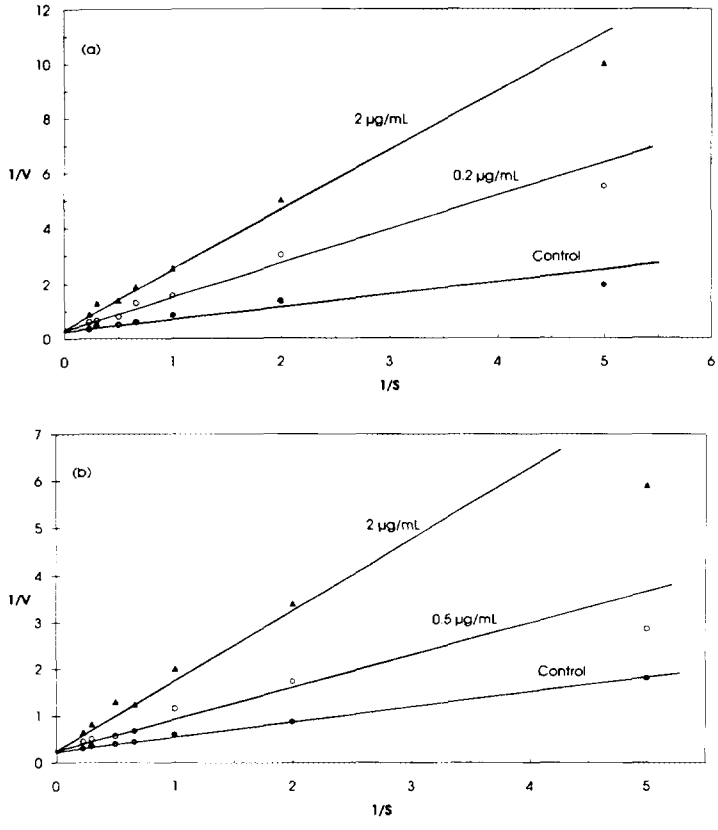

FIG. 6. Effect of substrate concentration on calystegin B inhibition of (a) β-glucosidase and (b) α-galactosidase. Incubations were as described in the text except that the amount of substrate was varied as indicated. The liberation of *p*-nitrophenol was determined and the data were plotted by the method of Lineweaver and Burk.

the alkaloids is an excellent inhibitor. Calystegins B_1 and B_2 both incorporate the structural configuration of calystegin A_3, which is itself a reasonably good inhibitor of β-glucosidase and α-galactosidase. In the castanospermine series, it has been found that an increasing degree of hydroxylation is associated with enhanced inhibitory potential (33). It is therefore reasonable to suppose that the additional hydroxyl groups, located at C-6 and C-4 respectively in calystegins B_1 and B_2, increase the level of inhibitory activity in comparison to calystegin A_3, without altering the specific enzymes that are inhibited.

The inhibition of glycosidases by calystegins A and B identifies the polyhydroxy-*nor*tropanes as a novel family of alkaloids possessing such activity. The calystegins are thus the third structural class of bicyclic polyhydroxy alkaloids with such properties to be discovered. While their bicyclic ring system invites comparison with the polyhydroxy indolizidine and pyrrolizidine alkaloid groups, the pyrrolidine and piperidine ring moieties, rather than possessing the nitrogen atom at the point of fusion, have conjunction points at positions α- to the nitrogen atom to give the bridged tropane structure. As a result, the heterocyclic nitrogen is secondary instead of tertiary in nature, with consequent similarities to deoxynojirimycin (**5**) and its stereoisomers. The fact that the additional hydroxyl group in calystegins B_1 and B_2 does not alter the specificity of enzymes inhibited but only the potency, suggests that the six-membered ring moiety is the structural feature essential for biological activity. However, the inhibition of both β-glucosidase and α-galactosidase does not permit definitive conclusions to be drawn with regard to the influence of hydroxyl group stereochemistry upon specific enzyme inhibition. Nevertheless, the addition of a new structural class of polyhydroxy alkaloids to those already identified provides significant information for the use of molecular modeling techniques in developing a predictive theory of glycosidase inhibition by such compounds. The calystegins may also prove to be useful additions to those inhibitors presently available for investigations of glycoprotein biosynthesis and degradation in cell cultures.

The biological role of the calystegins in the plant species which produce them is not understood at the present time. The alkaloids were not detected in the subterranean or aerial parts of 102 other plant species, encompassing 26 families, which were analyzed for their presence. The calystegins are produced in high yield in both the roots of naturally growing plants and in transformed root cultures grown *in vitro*. It has been shown in the laboratory that they are specifically catabolized by the soil microorganism, *Rhizobium meliloti* strain 41, providing a source of carbon and nitrogen which stimulates growth. More than 40 other rhizosphere bacteria, including other strains of *R. meliloti*, have been tested and found to lack the ability to utilize the calystegins as a nutritional source. The rare occurrence of both calystegin biosynthesis and catabolism, together with the high levels of these alkaloids in below-ground plant structures suggests that they influence rhizosphere ecology through nutritional selection. They are probably not directly involved in nitrogen fixation symbiosis. In addition to homoserine and the opines they constitute a third class of natural products associated with Rhizobiaceae nutritional factors. Their occurrence as normal metabolites of plants is in contrast to nutritional mediators that are generated by genetic transformation and symbiosis.

The establishment and maintenance of specific plant–bacterium relationships have been shown to be regulated by secondary metabolites elaborated by the host. Simple phenolic natural products such as acetosyringone induce transcription of *Ag. tumefaciens* virulence (*vir*) genes in wounded tissues (34) and some of the more complex phenolic metabolites exuded by legume roots, the flavonoids, induce transcription of nodulation (*nod*) genes in rhizobia (35). Other secondary metabolites have been shown in laboratory tests to selectively nourish certain soil bacteria. These include the amino acid derivatives called opines (36), homoserine (37), the aminocyclitol rhizopine (38), and the betaines (39). Such compounds may therefore serve as nutritional mediators in nature, selectively pro-

moting the growth of specific bacteria and consequently modifying the ecology of the rhizosphere. A soil bacterium that depends for carbon on root exudates, some of which may be in the form of sugars, might suffer from inhibition of its sugar-metabolizing enzymes in the presence of calystegins. Thus, bacteria such as *R. meliloti* strain 41 that degrade calystegins (Cac^+) would gain carbon and nitrogen from the calystegins, but would also gain access to the sugars that other soil bacteria (Cac^-) are unable to utilize. Knowledge of the biochemical mode of action of the calystegins may therefore provide an understanding of their role in the rhizobial ecology of the plants which produce them and possibly lead to control methods for members of the genera *Convolvulus* and *Calystegia* which are significant weed species in many areas of the world.

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